ECVAM Protocol for Rat Skin Transcutaneous Electrical Resistance: an *In Vitro* Assay for Assessing Dermal Corrosivity

Original Draft: July 1996
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NOTE: This protocol presents the standard operating procedure used in the ECVAM Skin Corrosivity Validation Study (1996/1997). ECVAM confirmed the accuracy of the SOP in October 2000, and this protocol was supplied by Dr. Andrew Worth of ECVAM via email on May 22, 2001.

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Rat Skin Transcutaneous Electrical Resistance (TER) Test

The corrosivity potential of a chemical may be predicted from its effects on the transcutaneous electrical resistance of rat skin and from its effects on the penetration of sulforhodamine B dye through the skin.

Objectives and Applications

TYPE OF TESTING	:	screening, replacement
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency
PURPOSE OF TESTING	:	hazard identification, classification and labelling

Proposed replacement for the *in vivo* method, the Draize rabbit skin corrosivity test, to be used for hazard identification and classification of corrosive potential to fulfil international regulatory requirements pertaining to the handling, packing and transport of chemicals.
When used in screening mode, the TER test is employed to predict corrosivity potential rather than the degree of corrosive effect (i.e. potency) (Fentem *et al.,* 1998).

Basis of the Method

Most international regulatory classification schemes define chemically induced dermal corrosion as full thickness destruction (necrosis) of the skin tissue, while some extend the definition of corrosion to include any irreversible alterations caused to the skin. The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals.

The determination of skin corrosion potential is therefore included in international regulatory requirements for the testing of chemicals, for example, in OECD testing guideline 404 (Anon., 1992); Annex V of Directive 67/548/EEC (Anon., 2000) and in the U.S. Code of Federal Regulations (Anon., 1991).

Corrosivity is usually determined *in vivo* using the Draize rabbit skin test (Draize *et al.,* 1944). The present test is based on the experience that transcutaneous electrical resistance (TER) measurements are believed to be of value in predicting severe cutaneous effects *in vivo*. The TER assay developed and evaluated by Oliver and coworkers (Barlow *et al.,* 1991; Oliver *et al.,* 1986; 1988; Oliver, 1990) has been used successfully as a routine in-house test for several years (Fentem *et al.,* 1998).

As an outcome of the ECVAM prevalidation study for protocol optimization, a second endpoint, dye binding (sulforhodamine B) has been added to reduce the number of false positive predictions encountered previously with surfactants and neutral organics.

Experimental Description

Endpoint and Endpoint Detection	:	- changes in transcutaneous electrical resistance (kΩ); - dye binding (sulforhodamine B) determined by optical density measurements;
Test System	:	isolated rat skin.

Liquid or solid test material is applied to the inner epidermal surface of discs of freshly isolated rat dorsal skin. After the exposure periods of 2 and 24 hours, the skin is washed and transcutaneous electrical resistance is measured. If the electrical resistance values are <5kΩ and the substance is a surfactant or neutral organic, then the sulforhodamine B dye is applied to the epidermal surface of each skin disc. The discs are washed and then subjected to a dye extraction procedure. The amount of dye extracted is determined from optical density measurements. The changes in the endpoints are then compared to HCl and H_2O, the positive and negative controls.

Test Compounds

A total of 60 test compounds, consisting of 11 organic acids, 10 organic bases, 9 neutral organics, 5 phenols, 7 inorganic acids, 4 inorganic bases, 3 inorganic salts, 8 electrophiles and 3 soaps/surfactants were tested in the ECVAM validation study. Details on the test compounds and test results are available from dbVas of the ECVAM SIS.

Prediction Model

Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a predetermined threshold level.

If the transcutaneous electrical resistance readings are ≤5kΩ at either of the contact periods, and the substance is a surfactant or neutral organic, then the dye penetration results are considered.

For detailed information see section 11, “Interpretation of results” of the present standard operating procedure.

Discussion

The TER assay is robust, requires inexpensive and readily available equipment, and can be performed by most laboratory personnel provided that care is taken during the critical steps of disc preparation and washing. The assay is inexpensive to perform in comparison with the three-dimensional tissue culture models and the CORROSITEX assay, and the technology is not protected by patent. These factors support the overall applicability of the TER assay in routine testing. The validation study has demonstrated the accuracy of the TER assay in identifying C and NC chemicals (Fentem *et al.*, 1998).

Status

The TER assay has been evaluated in intralaboratory and interlaboratory studies (Botham *et al.,* 1992; Oliver *et al.,* 1986, 1988), and it performed creditably in the prevalidation study conducted during 1993 and 1994 (Botham *et al.,* 1995). This method has been evaluated in the ECVAM Skin Corrosivity Validation Study conducted in 1996 and 1997 (Fentem *et al.,* 1998). Based on the outcome of the study, the ECVAM Scientific Advisory Committee (ESAC) concluded that the results obtained with the rat skin TER test in the "ECVAM Skin Corrosivity Validation Study" were reproducible. The test proved applicable to testing all the above reported chemical classes of different physical forms. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good.

ESAC unanimously endorsed the statement that the rat skin TER test was scientifically validated for use as a replacement for the animal test for distinguishing between corrosive and non-corrosive chemicals, and that this test was ready to be considered for regulatory acceptance (10^{th} meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 1998; Anon., 1998). The 27^{th} meeting of the Committee for Adaptation to Technical Progress of "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" agreed that the TER Test would form part of "Annex V method B.40. Skin Corrosion", February 2000 (Directive 2000/33/EC). Furthermore, this test is now under consideration for inclusion in the OECD Guidelines. Further details on the ECVAM Validation Study are available from dbVas of the ECVAM SIS.

Last update: May 2000

Procedure Details, July 1996*

RAT SKIN TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST

NOTE: This protocol presents the standard operating procedure evaluated in the ECVAM Skin Corrosivity Validation Study (1996/1997).

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* The accuracy of the SOP has been confirmed in October 2000.

1. INTRODUCTION AND OBJECTIVES

The purpose of this technique is to assess the degree of the skin corrosive potential of a test chemical *in vitro*. The results obtained from the transcutaneous electrical resistance (TER) measurements are believed to be of value in predicting severe cutaneous effects (degree of skin corrosive potential) *in vivo*. As a prelude to formal validation, the TER assay was evaluated in a prevalidation study (Botham *et al.*, 1995). Preliminary evaluation of the results indicated that the TER test required optimisation, to enable differentiation between different classes of corrosive materials, and to reduce the number of over-predictions (false positives). The results of this optimisation (Hadfield & Lewis, 1996; unpublished data), indicated that the modified electrical resistance test was able to differentiate between classes of corrosive materials (R35/R34) and, by the addition of a second endpoint, dye binding, was able to reduce the number of false positive predictions. The following protocol was therefore devised for use in the ECVAM international validation study on *in vitro* tests for skin corrosivity (Barratt *et al.*, 1998; Fentem *et al.*, 1998).

2. SAFETY PRECAUTIONS

Standard local safety precautions should be adopted. All materials should be handled in accordance with their potential hazards.

3. ANIMALS AND HUSBANDRY

20-23 day old Wistar rats are purchased for use in the test. Animals are acclimatised for a minimum of one night, depending on the day of delivery. On the day after arrival they are shaved and washed: animals are held securely and the dorsal flank hair is carefully removed with small animal clippers. The animals are then washed by careful wiping, whilst submerging the area in a one-litre volume of antibiotic solution (see following section 4). Animals are washed again on the third or fourth day following the first wash, and they are then used within 3 days (animals must

not be older than 31 days for pelt preparation).

4. PREPARATION OF ANTIBIOTIC SOLUTION

An antibiotic solution is prepared by adding streptomycin, penicillin, chloramphenicol and amphotericin B to 1 litre of luke-warm deionised water. The resulting antibiotic solution should contain the following concentrations: 8mgml^{-1} streptomycin; 800µgml^{-1} penicillin; 10µgml^{-1} chloramphenicol; and 10µgml^{-1} amphotericin B. Streptomycin, penicillin, chloramphenicol and amphotericin B are available from standard laboratory suppliers. It is also acceptable to use mixtures of antibiotics containing glutamine which are commercially available. Appropriate inhalation safety procedures should be followed when handling antibiotics.

5. PREPARATION OF SKIN AND MOUNTING ON *IN VITRO* APPARATUS

Animals are humanely killed by inhalation of a rising concentration of CO_2 followed by cervical dislocation. The dorsal skin of each animal is then removed and stripped of excess fat by carefully peeling it away from the skin by using the thumb and forefinger covered with paper towel. The pelt is placed over the end of a 10 mm diameter polytetrafluoroethylene (PTFE) tube ensuring that the epidermal surface is in contact with the tube. A rubber 'O' ring is press-fitted over the end of the tube to hold the skin in place, and excess tissue is trimmed away with a scalpel blade. Tube and 'O ' ring dimensions are shown in Figure 3. The rubber 'O' ring is then carefully sealed to the end of the PTFE tube with petroleum jelly (or soft paraffin wax), applied with a scalpel blade. The tube is supported by a spring ("Terry") clip inside a plastic receptor chamber containing 10ml of magnesium sulphate solution (154mM; see Figure 1). The PTFE tube is uniquely numbered with a label prior to test substance application.

Skin discs of approximately 0.79cm^2 can be obtained from any number of animals. However, the viability of each pelt must be assessed prior to use in the test by using the following method: two discs are taken from each pelt and prepared as described above. Electrical resistance measurements are then taken for each disc (see section 7). Both discs must produce resistance values of ≥10kΩ. The two discs are then discarded and the remainder of the pelt is used in the test. If both discs fall below the 10kΩ threshold, the pelt is discarded. If one disc falls below this threshold, another is tested; if this also falls below the threshold, the pelt is discarded. If the disc produces a TER measurement of ≥10kΩ, the pelt can be used in the test.

PTFE tubes and rubber 'O' rings are available from IMS, Dane Mill, Broadhurst Lane, Congleton, Cheshire CW12 1LA, UK.

6. TEST CHEMICAL APPLICATION AND REMOVAL

A measured volume of liquid test material (0.15ml) is applied to the inner epidermal surface (see Figure 1). When using solid test materials, a sufficient amount of solid material is applied to the surface of the disc ensuring that the whole surface of the epidermis is covered. Deionised or distilled water (0.15ml) is then added on top of the solid material and the tubes are shaken. Three skin discs are used for each time point per chemical. Test chemicals are applied for contact periods of 2 and 24 hours. After the required contact time, the test chemical is removed by washing with a jet of tap water, at room temperature, for approximately 10 seconds or until no further test material can be removed.

Control substances for the TER test and the dye binding assay:

Positive	-	36% HCI
Negative	-	DH_2O

All to be tested at the 24-hour contact period only.

Test substances should have maximum contact with the skin. For some solids this may be achieved by warming up to 30°C to melt the test substance, or by grinding to produce a granular material or powder.

Where measured test substance TER values are higher than the negative (water) control values (for example, waxy solids which may become liquids at approximately room temperature), the skin surface can be washed with water at up to 37°C. The skin should be visually inspected to determine if the skin is coated with test substance. The TER value should then be re-measured. If the value is less than or equal to the upper limit of the negative (water) control range, and if the skin disc appears to be free of residue, it can be accepted. If the TER value does not reduce to the upper limit of the negative control range after washing with the warm water, the disc should be rejected.

7. TRANSCUTANEOUS ELECTRICAL RESISTANCE MEASUREMENTS

The transcutaneous electrical resistance is measured using an AIM electronic databridge 401 or 6401 (available from H. Tinsley and Co., 275 King Henry's Drive, New Addington, Croydon, Surrey CR0 0AE, UK).

Prior to measuring the electrical resistance, the surface tension of the skin is reduced by adding a small volume of 70% ethanol sufficient to cover the epidermis. After approximately 3 seconds, the ethanol is removed by inverting the tube. The PTFE tube is then replaced in the receptor chamber and the tissue is hydrated by the addition of 3ml of magnesium sulphate solution (154mM) to the inside of the PTFE tube; any air bubbles are dislodged by slight tapping.

The stainless steel electrodes of the databridge are then placed on either side of the skin disc to take the resistance measurement in kΩ/skin disc (see Figure 2). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 3. The inner (thick) electrode clip is rested on the top of the PTFE tube during resistance measurement, to ensure that a consistent length of electrode is submerged in the $MgSO_4$ solution. The outer (thin) electrode is positioned inside the receptor chamber, so that it rests on the bottom of the chamber. The distance between the bottom of the Terry clip and the bottom of the PTFE tube is set at 7.0 cm, to reduce the variability of resistance measurements between individual skin discs, which is influenced by the distance between the electrodes. The electrical resistance is then recorded from the databridge display.

If the reading falls above 20kΩ this may be due to the test material coating the epidermal surface of the skin disc. Removal of this coating can be performed by holding a gloved thumb over the end of the tube and shaking it for approximately 10 seconds; the $MgSO_4$ solution is then poured away. If any test material is present it may be seen as a residue in the $MgSO_4$ solution. The transcutaneous electrical resistance of the skin is then measured as described previously.

8. SULFORHODAMINE B DYE APPLICATION AND REMOVAL

If the electrical resistance values are ≤5kΩ at the 2- and/or 24-hour contact periods, an assessment of dye penetration is carried out on the 24-hour contact period tissues. If the skin disc was punctured during the jet washing procedure to remove the test chemical, then that particular tube is excluded from further testing.

150µl of a 10% (w/v) dilution of sulforhodamine B dye in DH_2O is applied to the epidermal surface of each skin disc for 2 hours. To remove any excess/unbound dye, the skin discs are then jet-washed with tap water at room temperature for approximately 10 seconds (or until the water runs clear). Each skin disc is carefully removed from the PTFE tube and placed in a 20ml scintillation vial containing 8ml of deionised water. The vials are agitated gently for 5 minutes to remove any further excess/unbound dye. This rinsing procedure is then repeated. Each skin disc is removed and placed into another 20ml scintillation vial containing 5ml of 30% (w/v) sodium dodecyl sulphate (SDS) in DH_2O and is incubated overnight at 60°C.

After incubation, each skin disc is removed and discarded and the remaining solution is centrifuged in a 15ml centrifuge tube at 1000rpm for 8 minutes at 21°C (relative centrifugal force ≈ 175g). A 1ml sample of the supernatant is then placed into another 15ml centrifuge tube and diluted 1 in 5 (v/v) (i.e. 1ml + 4ml) with 30% (w/v) SDS in DH_2O. The optical density of the solution is determined at 565.5nm and the results are recorded.

Sulforhodamine B (90% dye content) and SDS are available from Sigma Chemical Company, Poole, UK.

9. FURTHER INFORMATION

Experience with the TER assay has shown that there are two critical stages. Experienced users pay particular attention to: a) skin disc preparation, ensuring removal of all fatty tissues and a complete seal of the skin on the PTFE tube; b) washing of the disc to remove as much of the test substance as possible. Residues of test substance remaining on the skin may affect the resistance values (for example, waxy substances, which solidify on the skin's surface). The positive controls TER values can drift with time (within days) if the samples are not fresh aliquoted from the stock acid maintained according to the storage recommendations on the label.

10. CALCULATION OF DYE CONTENT/DISC

The dye content, in µg/disc, is calculated from the optical density values as follows:

Sulforhodamine B dye molar extinction coefficient = 8.7×10^4,
Molecular Weight = 580,
No correction for the purity of the dye is made,
Optical density = 0.973,

$$\frac{0.973 \leftrightarrow 10^{-4}}{8.7} = 0.112 \leftrightarrow 10^{-4} = 11.2 \leftrightarrow 10^{-6} = 11.2 \mu M = 11.2 \mu\text{mol/l}$$

$$11.2 \leftrightarrow 580 \leftrightarrow 10^{-6} = 6496 \leftrightarrow 10^{-6}\ \textbf{\textit{g/l}} = 6.496 \leftrightarrow 10^{-3}\ \textbf{\textit{g/l}}$$

Dye was extracted into 5ml of solvent:

$$\frac{6.496 \times 10^{-3}}{200} = 0.325 \times 10^{-4}\ g/l = 32.5 \times 10^{-6}\ g/l$$

Solution was diluted 1 in 5 (v/v):

$$32.5 \times 10^{-6} \times 5 = 162.5 \times 10^{-6} = 162.5 \mu g/disc$$

The sulforhodamine B dye content is determined for each skin disc. A mean dye content is then calculated for the three skin discs at 24 hours. If a skin disc is punctured during the washing procedure used to remove the dye, then the individual dye content is recorded but it is excluded from the calculation of the mean.

All results are recorded on the data sheet shown in Appendix 1.

11. INTERPRETATION OF RESULTS

a) Results are accepted on condition of adherence to the ranges given below. If the positive and negative control results for the experiment do not fall within the accepted ranges, the data on the test substance cannot be interpreted and the experiment must be repeated.

Dye binding assay		TER assay	
36% HCl positive control range (µg/disc)	Distilled water negative control range (µg/disc)	36% HCl positive control range (kΩ)	Distilled water negative control range (kΩ)
40 - 100	15 – 35	0.5 - 1.0	10 - 25

b) If the transcutaneous electrical resistance readings obtained for all test substance contact periods are >5kΩ, then the substance is classified as non-corrosive.

c) If the transcutaneous electrical resistance readings are ≤5kΩ after a 2-hour contact period, and the substance is not a surfactant or neutral organic, then the substance is classified as corrosive (R35).

d) If the transcutaneous electrical resistance readings are ≤5kΩ after a 24-hour contact period (but >5kΩ after 2 hours contact), and the substance is not a surfactant or neutral organic, then the substance is classified as corrosive (R34).

e) If the transcutaneous electrical resistance readings are ≤5kΩ at either of the contact periods, and the substance is a surfactant or neutral organic, then the dye penetration results are considered.

f) If the mean disc dye content is ≥mean disc dye content of the 36% HCI positive control obtained concurrently in the experiment at the 24-hour contact period, then the substance is a true positive and is therefore classified as corrosive (R34).

g) If the mean disc dye content is < mean disc dye content of the 36% HCI positive control obtained concurrently in the experiment at the 24-hour contact period, then the substance is a false positive and is therefore classified as non-corrosive.

A flow diagram for interpretation of the results is attached.

Figure 1

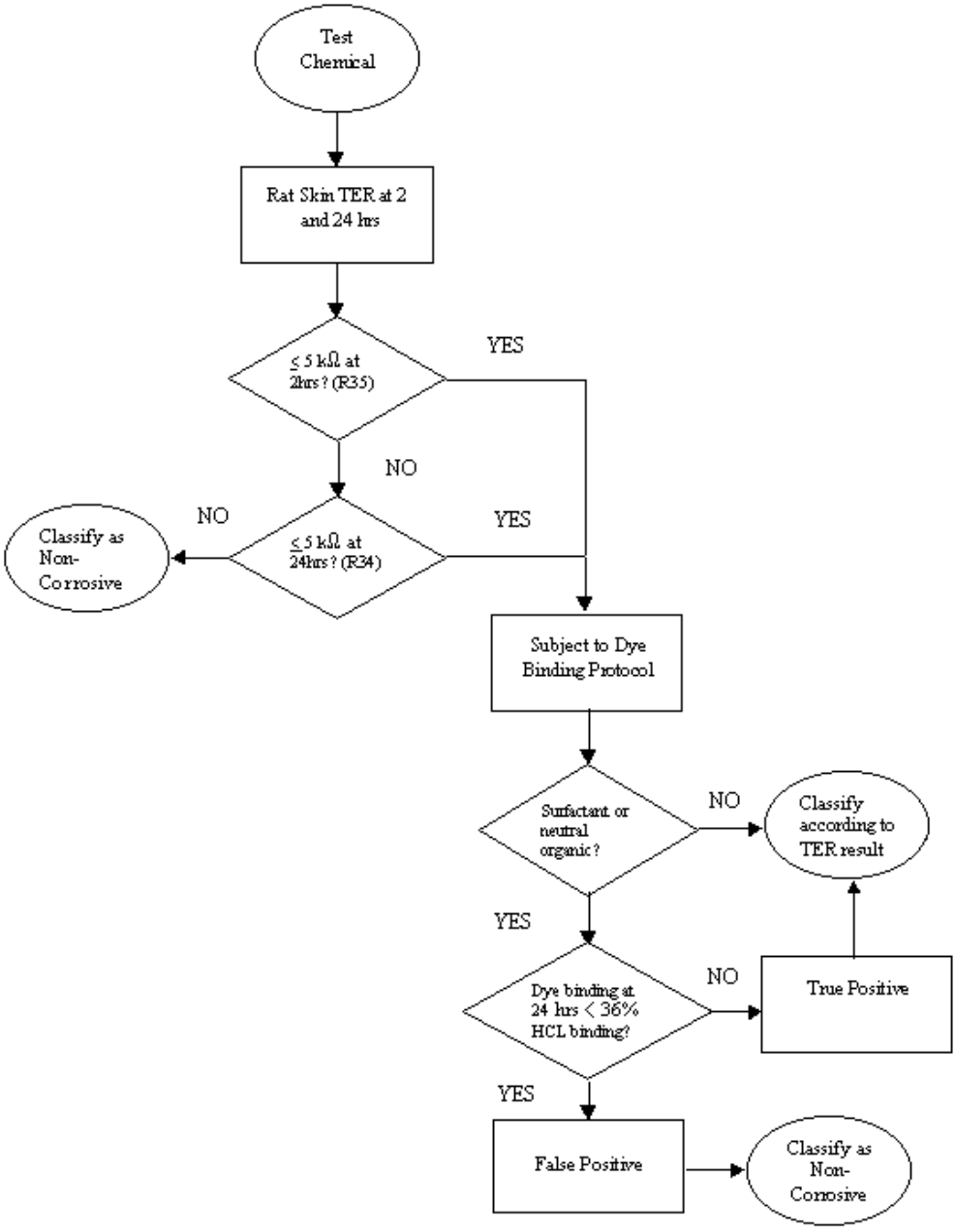

Test Chemical
Rat Skin TER at 2 and 24 hrs
≤ 5 kΩ at 2hrs? (R35)
YES
NO
≤ 5 kΩ at 24hrs? (R34)
NO
YES
Classify as Non-Corrosive
Subject to Dye Binding Protocol
Surfactant or neutral organic?
NO
Classify according to TER result
YES
Dye binding at 24 hrs < 36% HCL binding?
NO
True Positive
YES
False Positive
Classify as Non-Corrosive

Figure 2

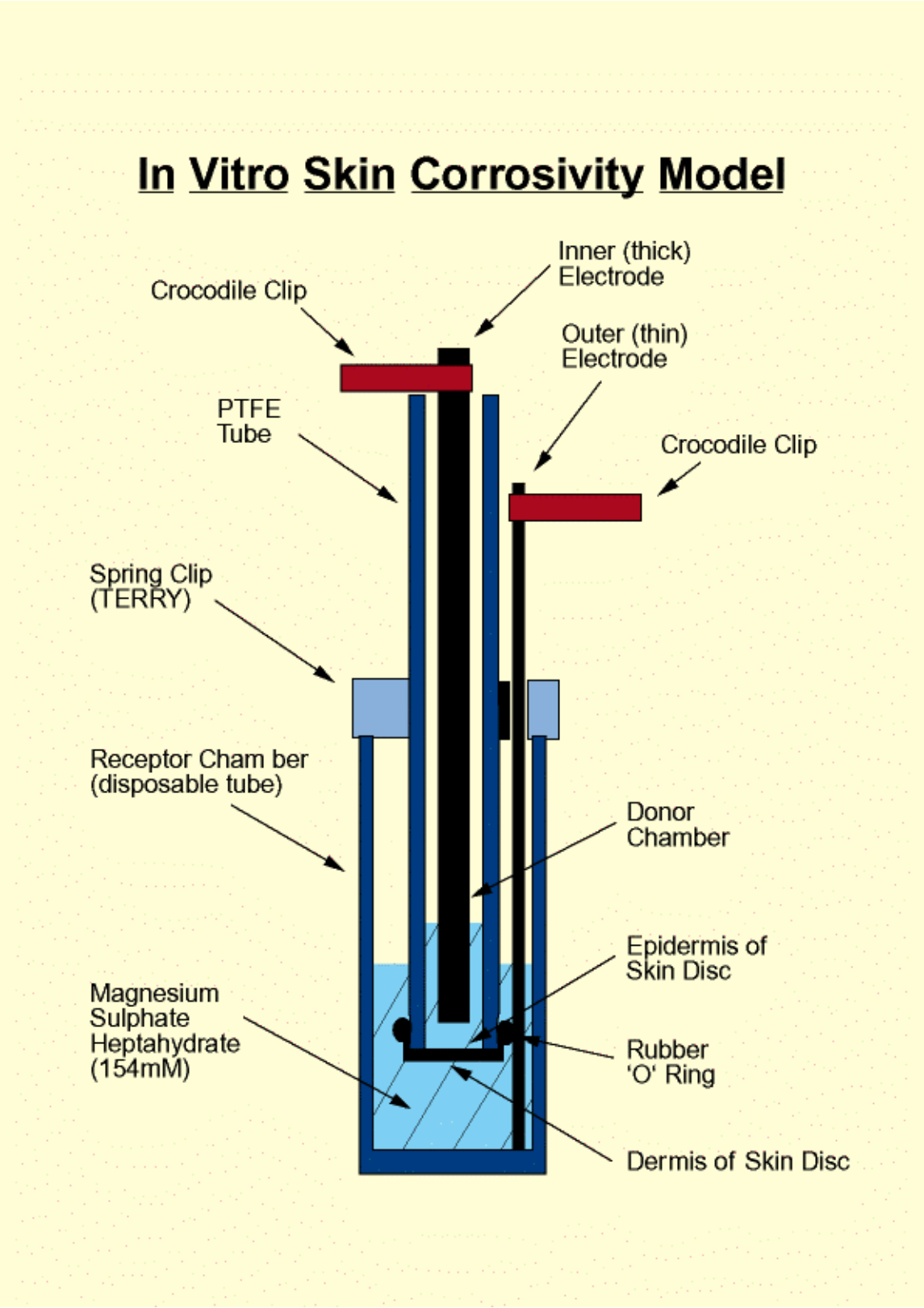
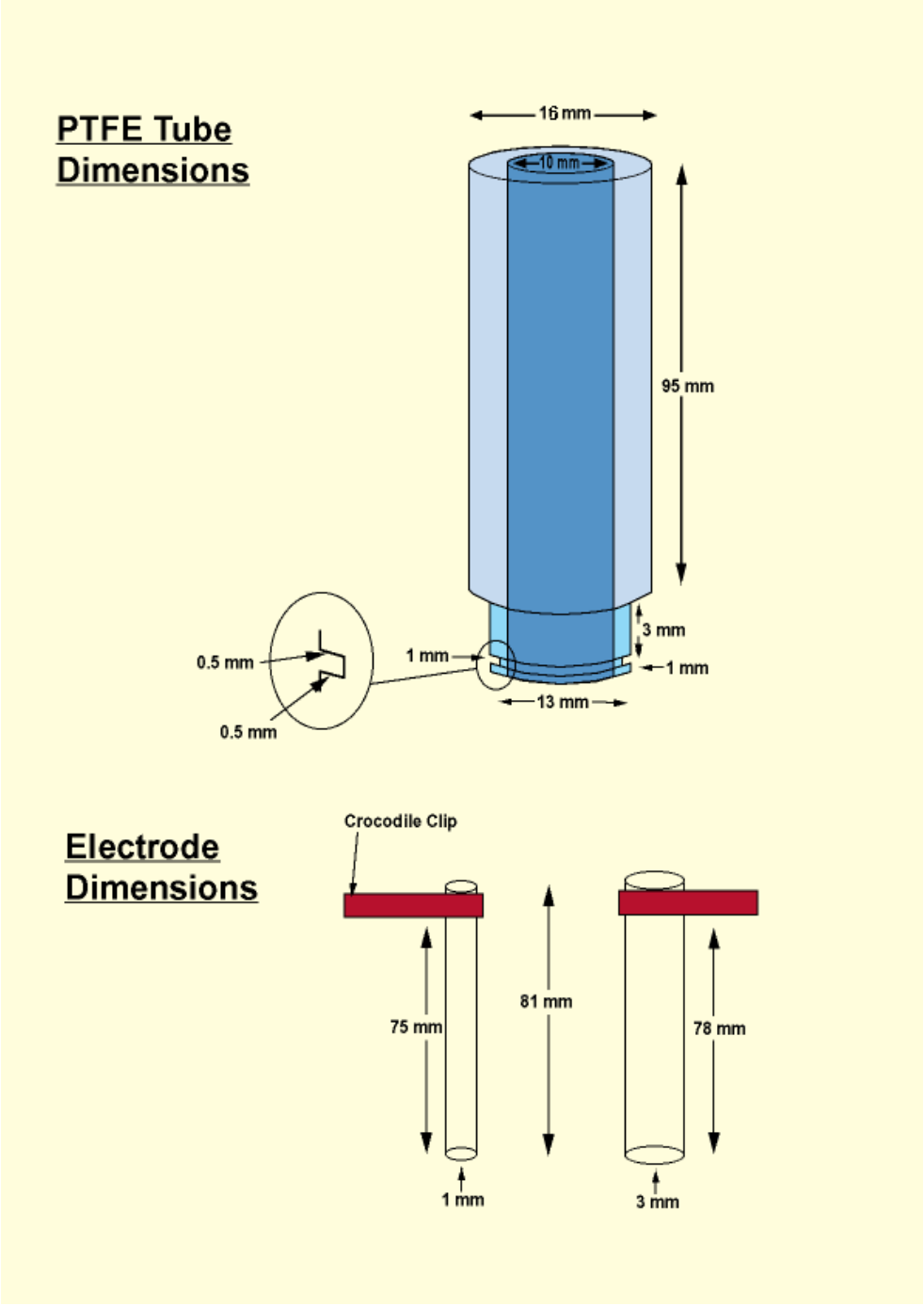

In Vitro Skin Corrosivity Model
Inner (thick) Electrode
Crocodile Clip
Outer (thin) Electrode
PTFE Tube
Crocodile Clip
Spring Clip (TERRY)
Receptor Cham ber (disposable tube)
Donor Chamber
Epidermis of Skin Disc
Magnesium Sulphate Heptahydrate (154mM)
Rubber 'O' Ring
Dermis of Skin Disc

Figure 3


PTFE Tube Dimensions
16 mm
10 mm
95 mm
3 mm
1 mm
13 mm
0.5 mm
1 mm
0.5 mm
Electrode Dimensions
Crocodile Clip
75 mm
81 mm
78 mm
1 mm
3 mm

APPENDIX 1: ECVAM Validation Study

Transcutaneous Electrical Resistance Test/Dye Binding - Results Summary Sheet

Laboratory Study Number: ____________ Laboratory Name: ____________ Experiment Number ____________

Test Chemical and appearance	Contact Time (hours)	Tissue number	Electrical Resistance (kohms)	Mean TER ± S.D. (kohms)
	2			
	24			

Dye Contact Time (hours)	Optical Density at 565.5 nm (diluted 1 in 5)	Dye Concentration (µg/disc)	Mean Dye Concentration ± S.D. (µg/disc)	10% HCl Positive Control Mean Dye Concentration ± S.D. (µg/disc)	Classification	
2					R35	PG I
					R34	PG II/III
					NC	NC

Signature: ____________ Date: ____________

Checked by: ____________ Date: ____________

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